

Heart Rate Regulation by G Proteins Acting on the Cardiac Pacemaker Channel

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Heart rate is determined by pacemaker currents, of which the most important is the hyperpolarization-activated current I_f . Heart rate and I_f are increased by β -adrenergic agonists and decreased by muscarinic agonists released from cardiac sympathetic and vagal nerves, respectively. The hypothesis that the receptors for each agonist are directly coupled to I_f channels by G proteins was tested. Under substrate-free conditions, preactivated G protein G_s stimulated and preactivated G protein G_o inhibited I_f channels of sinoatrial node pacemaker cells. These effects were mimicked by the corresponding preactivated α subunits of the G proteins. Unexpectedly, the two G proteins acted simultaneously, with G_o being the more potent. This result may explain in molecular terms the classical observation in cardiac physiology, that vagal inhibition of heart rate is much greater on a background of sympathetic stimulation.

THE MAMMALIAN HEARTBEAT ARISES from spontaneous, pacemaker currents in sinoatrial (S-A) node cells and is neurally regulated by the stimulatory, β -adrenergic effects of the transmitter norepinephrine and the inhibitory, muscarinic cholinergic effects of the transmitter acetylcholine released from the cardiac sympathetic and vagus nerves, respectively. The key pacemaker current is the hyperpolarization-activated current I_f (1, 2). Muscarinic agonists decrease and β -adrenergic agonists increase I_f , and it is proposed that G proteins act upon adenylyl cyclase (AC) to change the phosphorylation of I_f channels (3, 4).

Since the heart rate can change from second to second (5), if phosphorylation and dephosphorylation of the channel cause this change, these reactions must occur at subsecond rates. This is unlikely (6), and an alternative hypothesis has been proposed in which G proteins couple these receptors to pacemaker channels by faster, more direct, membrane-delimited pathways (7). A necessary condition for the direct coupling hypothesis would be that G proteins directly regulate I_f channels in S-A node cells. We report here that the G protein G_s preactivated with guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) (G_s^*) or its preactivated α subunit (α_s^*) (8) stimulated I_f channels in inside-out (IO) membrane patches excised from S-A node cells under substrate-free conditions. The preactivated G proteins G_s^* and G_i^* (8, 9) or their respective preactivated α subunits α_s^* and α_i^* inhibited I_f channels with G_o^* being more potent. G_s^* and G_i^* or their α subunits seem to act simultaneously

on an apparently shared set of I_f channels. The simultaneous action may provide a molecular explanation for why vagal inhibition of heart rate is greater in the presence of cardiac sympathetic nerve stimulation (synergistic effect) and why cardiac sympathetic stimulation of heart rate is smaller in the presence of cardiac vagal nerve stimulation (antagonistic effect) (10).

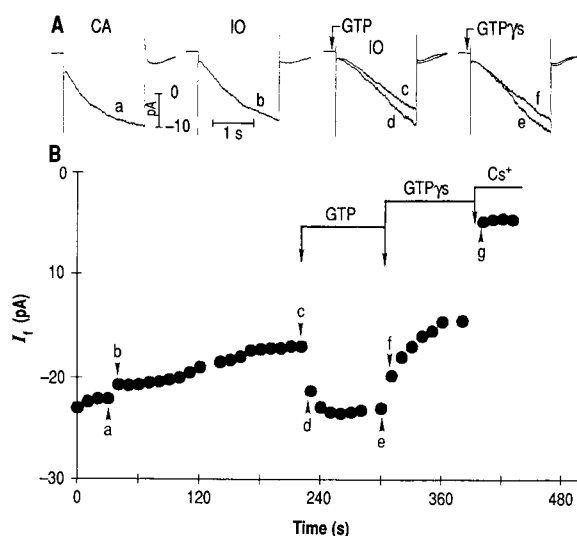
The current I_f can be recorded from cell-free, IO membrane macropatches (11). If we assume that single-channel conductance is 1 pS, patch I_f originated from about 200 channels (12). While still cell-attached (CA), patch I_f had the same waveform and voltage-dependence as whole-cell (WC) I_f (11) (Fig. 1A, trace a) and ran down slowly with time. Upon excision to the IO configuration, patch I_f fell immediately and then resumed its slow decline (Fig. 1, A, trace b, and B). To test whether autonomic receptors were coupled to I_f by G proteins in these cell-free patches, we added the β -adrenergic agonist isoproterenol (iso) to the pipette solution and changed guanosine triphosphate (GTP) in the bath. Concentration jumps of GTP increased I_f (parts c and d of Fig. 1, A and B). The effects were reversible and Mg^{2+} -dependent ($n = 4$). The time course could be determined by the difference in current between the traces before and immediately after the concentration jump of GTP. This time course indicated a delay of 50 ms and a time constant of 800 ms. Both rate and magnitude of the increase in I_f varied directly with the concentration of GTP; the increase could be as great as 50% ($37.3 \pm 9\%$, mean \pm SD; $n = 3$). With carbachol (carb) in the pipette, the same series of tests produced almost identical results except for a change in sign (Fig. 2A); I_f was reduced by

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$31 \pm 8\%$ ($n = 3$). The absolute requirements for GTP and Mg^{2+} implicated G proteins in the coupling of β -adrenergic and muscarinic receptors to I_f channels. Effects due to trapping of cytoplasmic substrate were excluded by adding adenylyl-5'-yl imidodiphosphate [AMP-P(NH)P] (2 mM) to the bath solution; the responses were unaltered, excluding channel phosphorylation as a mechanism. Taken together, the results supported the hypothesis that autonomic receptors were coupled to I_f by direct G-protein pathways.

Indirect pathways are thought to link β -adrenergic receptors and muscarinic receptors to I_f channels (13). In these cases, different G proteins were presumed to act on AC and, subsequently, on a shared cytoplasmic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase pathway. Our experiments suggested that there are two direct G protein pathways to I_f channels. As a further test of this hypothesis, we increased patch I_f with iso (1 μ M) in the pipette and GTP (100 μ M) in the bath and then replaced GTP with GTP γ S (100 μ M) (Fig. 1). GTP γ S produced no immediate increase in current, indicating that a maximum iso effect had been attained with GTP. Instead, patch I_f fell and continued to fall with further pulses (parts e and f of Fig. 1, A and B). When GTP γ S was added in the absence of any agonist, I_f also fell, but not as dramatically (5 to 10% at 100 μ M; $n = 8$), possibly from offsetting effects due to activation of endogenous inhibitory and stimulatory G proteins. We interpreted these results to indicate that G proteins inhibitory to I_f were dominant over stimulatory G proteins.

Fig. 1. Membrane-delimited G protein coupling of autonomic receptors to patch I_f channels. Concentration steps of GTP (100 μ M) and GTP γ S (100 μ M) (arrows) were applied with iso (1 μ M) in the patch pipette. Patch I_f was produced by hyperpolarizing test pulses of 2-s duration applied at 0.1 Hz. Holding potential was -30 mV, and test potential was -130 mV. Patch I_f never reached a steady state even with pulses of 10 s. Pulses longer than 2 s accelerated the rundown. (A) Patch I_f recorded in the CA mode (trace a) and after excision to cell-free, IO mode (trace b). Patch I_f is shown immediately before (trace c) and during the next (trace d) voltage-clamp pulse 100 ms after a step application of GTP. The effect of a step application of GTP γ S is shown by comparing the patch I_f before (trace e) and immediately after the step (trace f). The concentration steps were timed to deliver GTP γ S to the membrane patch 100 ms before the next voltage-clamp pulse. (B) The time course of peak I_f for the entire experiment shown in (A). Arrowheads show where the traces a through f in (A) were taken. K^+ in bathing solution was replaced by Cs^+ at (trace g).



Inhibition was not complete, and isosmotic substitution of Cs^+ for K^+ produced a further block of patch I_f (Fig. 1B, part g).

Our results supported a hypothesis in which different heterotrimeric G proteins directly regulate I_f channels as they do other ion channels (14). As a straightforward test, we added G_i^* , G_o^* , or G_s^* to patches containing I_f channels. G_o^* at picomolar concentrations reduced patch I_f (Fig. 2B). The effect was not apparent immediately but became apparent at the second test pulse and persisted during subsequent pulses. Exogenous G_o^* inhibited patch I_f more slowly than did GTP in the presence of carb, which acted in less than 1 s (15). Unlike the readily reversible

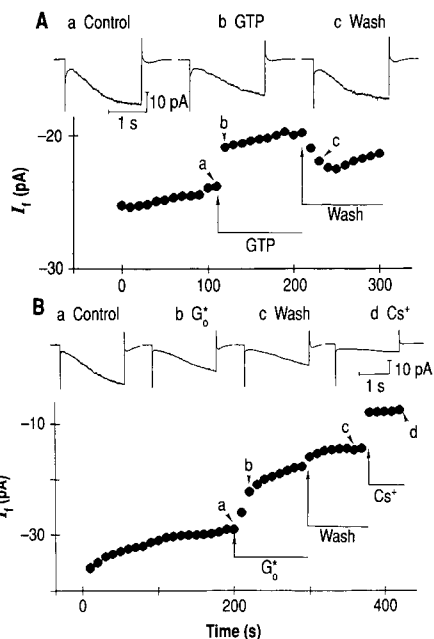


Fig. 2. Comparison of reversible inhibition of patch I_f produced by carb plus GTP (A) with irreversible inhibition produced by G_o^* (B). Patch I_f was produced by hyperpolarizing clamp pulses of 2-s duration at 0.1 Hz. Holding potential and test potential were -30 and -130 mV, respectively. (A) Time course of peak patch I_f after a concentration step of GTP from 0 to 100 μ M (arrow) and after wash out with GTP-free solution (arrow). (B) Time course of patch I_f after concentration step of G_o^* (100 pM), during wash out with G_o^* -free solution (arrow) and after addition of Cs^+ (arrow). K^+ in bathing solution was replaced by Cs^+ . Patch I_f currents at indicated times are shown above in (A) and (B).

inhibition produced by GTP in the presence of carb (Fig. 2A), the inhibition produced by G_o^* was irreversible, as expected from the effects of preactivated G proteins on other effectors (14) (Fig. 2B), and could not be overcome by prolonged washing for as long as 5 min ($n = 3$). Like the carb plus GTP block, the G_o^* block was incomplete, and Cs^+ produced a further reduction in patch I_f . To test the effects of different concentrations of G_o^* , we measured the magnitude of the inhibition at 2 to 3 min after a step change. The effects were apparent at concentrations as low as 10 pM and became greater at higher concentrations, reaching a maximum between 80 and 160 pM. The magni-

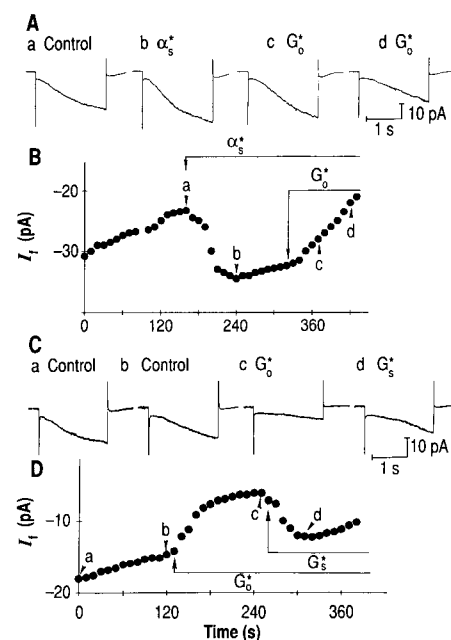


Fig. 3. The α_s^* subunit and G_o^* act simultaneously on patch I_f channels. Hyperpolarizing clamp pulses of 2-s duration were applied to -130 mV from a holding potential of -30 mV, at 0.1 Hz. α_s^* was added at the first arrow in (B) and G_o^* was added at the second arrow. The time course of peak patch I_f for the entire experiment is shown in (B), and the individual patch I_f currents for the times indicated in (B) are shown above in (A). In another experiment shown in (C) and (D), preactivated G proteins were added in the opposite sequence.

tude was normalized to the peak effect and, when plotted as a function of concentration, the curve had a mean inhibitory constant (IC_{50}) of about 50 pM ($n = 6$). In this way, the effects of G_o^* and G_i with type 3 α subunit ($G_{i\alpha-3}^*$) (16) were compared. G_o^* was about ten times as effective as $G_{i\alpha-3}^*$ which had an IC_{50} of about 500 pM ($n = 5$). Hence G_o , which is the dominant G protein in brain, where it can gate specific types of K^+ channels directly (9), may in S-A node cells be a pertussis toxin-sensitive substrate (17) and may couple muscarinic receptors to I_f .

A corollary of our hypothesis was that G_o^* should increase I_f . G_s^* or α_s^* increased patch I_f (Fig. 3A, part b) and, in the case of G_s^* , maximum increases were produced at concentrations of 80 to 160 pM ($23 \pm 9\%$; $n = 7$). Following stimulation, rundown resumed its slow course, but patch I_f was higher than before α_s^* application (Fig. 3B). Like the effects of G_o^* , the effects of G_s^* or α_s^* were irreversible. Prolonged washing up to 5 min with control bath solutions did not reverse the increased patch I_f . The effects of the different preactivated G proteins were mimicked by the respective preactivated α subunits ($n = 5$ for α_s^* ; $n = 8$ for α_o^*). In all cases with preactivated holo-G protein or α subunits, there were no requirements for GTP, Mg^{2+} or ATP, cAMP, and cAMP-dependent protein kinase at concentrations described in (13). Hence, the G^* proteins or their α^* subunits produced their effects on patch I_f independently from any of the usual second messengers. By contrast to the results with α^* subunits, dimeric $\beta\gamma$ subunits at much larger concentrations of 1 to 2 nM had no effect ($n = 8$) (18).

The results with G_o^* and G_s^* and the experiments with agonists and GTP γ S (Fig. 1) led to the prediction that G_o^* and G_s^* should converge upon I_f channels. We tested this first by applying G_o^* or α_o^* and then applying G_s^* . Both G proteins were applied at concentrations that produced maximal effects. The α_s^* subunit produced an increase in patch I_f , and the subsequent addition of G_o^* produced a decrease. The decrease was not due to dissociation of α_s^* , because after G_o^* application the currents were not reduced to the levels present before addition of α_s^* . Similar results were observed in two additional experiments. Applying the preactivated G proteins in the opposite order produced the opposite sequence of events (Fig. 3, C and D; $n = 4$). Since the effect on patch I_f of either preactivated G protein was irreversible and the combined effects were not competitive, both G proteins were probably acting simultaneously, if we assume one set of I_f channels. However, G_s and G_o may have regulated two independent sets of I_f channels. This is considered unlikely for the

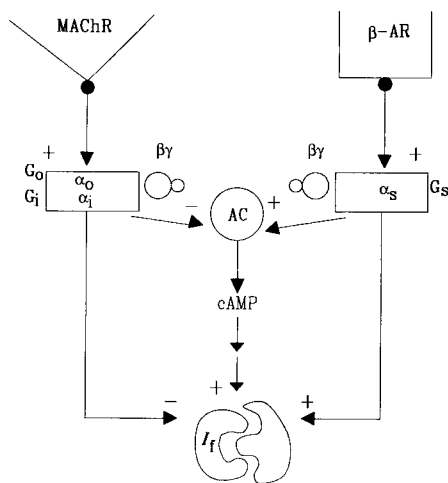


Fig. 4. G protein regulation of I_f by direct and indirect pathways from muscarinic (MACHR) and β -adrenergic (β -AR) receptors. AC, adenylyl cyclase.

following reasons: First, G_o^* at 100 pM produced decreases of $58 \pm 7\%$ in I_f after stimulation by supramaximal concentrations of α_s^* or G_s^* ($n = 3$) and decreases of $37 \pm 17\%$ in controls ($n = 6$). If there were two independent populations of I_f channels, the effects of G_o^* should have been smaller, not larger, after stimulation by G_s^* . Second, GTP γ S produced a large reduction in I_f after stimulation by iso plus GTP (Fig. 1) ($n = 4$) but no discernible stimulation of I_f after inhibition produced by carb plus GTP ($n = 7$). If there were two independent sets of I_f channels, then stimulation should have occurred.

The minimum G protein network required to account for the convergence of muscarinic and β -adrenergic agonist effects on I_f is shown in Fig. 4. The α subunits can act on I_f directly or indirectly by way of AC, and $\beta\gamma$ subunits (arrows not drawn) may be inhibitory to the α effects on AC (19). We have not included mutually inhibitory $\beta\gamma$ effects on I_f , although, by analogy with AC, they might be anticipated.

These experiments rule out $\beta\gamma$ subunits as mediators of G protein effects on I_f channels since, for the case of human erythrocyte G_o^* and G_i^* , the same $\beta\gamma$ must produce stimulation and inhibition of I_f simultaneously. For the same reasons, an effect through phospholipase A_2 , arachidonic acid, and a lipoxigenase metabolite reported for muscarinic atrial K^+ channels (20) can be excluded. Furthermore, cardiac myocytes do not have lipoxigenase activity (21). Our experiments suggest that one effector, the I_f channel, is regulated by two G proteins at distinct sites (22). The changes in I_f channels produced by G_o and G_s may explain in molecular terms the effects on heart rate produced by the combined stimulation of the cardiac

sympathetic and vagus nerves. The dominance of the vagal effect (10) is reflected by the dominance of the inhibitory G protein activated by GTP γ S (Fig. 1). Direct G protein coupling between autonomic receptors and I_f channels in cardiac pacemaker cells may account for the ability of the nervous system to produce its effects within a single heartbeat; indirect coupling via second messengers can account for more persistent effects.

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11. Single S-A node cells were isolated from albino rabbits by enzymatic dissociation [A. Yatani and A. M. Brown, *Am. J. Physiol.* **258**, 1947 (1990)]. Currents from membrane patches were recorded by the patch-clamp method with a List EPC7 amplifier [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfluegers Arch.* **391**, 85 (1981)]. Patch pipettes had resistances of 5 to 10 megohms. Currents were low pass-filtered (-3 dB) at 0.5 to 1.0 kHz and digitized at 1 to 2 kHz. Freshly isolated cells were placed in a chamber containing intracellular-like bathing solution: 110 mM potassium aspartate; 20 mM KCl; 2 mM $MgCl_2$; 5 mM EGTA; and 5 mM Hepes, pH 7.3, with tris base. For Mg^{2+} -free bathing solution, EGTA was replaced by EDTA. To exclude channel phosphorylation, AMP-P(NH)P (2 mM) was present in all experiments unless otherwise noted. Other nucleotides were added to the bathing solution when required. Nucleotides were from Boehringer Mannheim; other chemicals are from Sigma. The patch pipette solution was 70 mM NaCl; 70 mM KCl; 1 mM $MgCl_2$; 1.8 mM $CaCl_2$; 2 mM $MnCl_2$; 1 mM $BaCl_2$; and 5 mM Hepes, pH 7.3, with tris base. The catalytic subunit of cAMP-dependent protein kinase II (C subunit) was activated with 0.5 M dithiothreitol. All experiments were done at room temperature (20° to 22° C). All test agents were applied by the concentration-clamp method, which after a dead time of 50 ms changed solutions at the membrane within 10 ms [N. Akaike et al., *J. Physiol. (London)* **379**, 7 (1986); (7)].

12. DiFrancesco (2) reported a single-channel conductance of about 1 pS. Patch I_f was about 20 pA at -120 mV and had an extrapolated null potential of -20 mV. Assuming an open probability of 1.0, the channel density was about 200 per macropatch.
13. We have confirmed, in excised patches, the results of DiFrancesco and Tromba (4) in WC experiments that phosphorylation increases I_f . When a solution of ATP (2 mM), cAMP (100 μ M), and cAMP-dependent protein kinase (1 μ g/ml) was added to the bath, patch I_f was increased by $62.5 \pm 15\%$ ($n = 4$). This phosphorylating solution was ineffective when it was Mg^{2+} -free ($n = 6$).
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22. It is possible that two different subunits of I_f could each be regulated by a different G protein. In this case the allosteric changes on I_f would be produced by the subunit proteins rather than the G proteins themselves. Nonetheless, the changes on I_f would be allosteric.
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